

# Chapter 7

## Profiling of Urine Using ProteinChip® Technology

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### Abstract

Urine is an extremely valuable sample type for biomarker discovery due to the non-invasive collection and the relatively low protein content, which makes detection of perturbations associated with disease easier. SELDI-TOF analysis is ideally suited for analysis of urine since the chromatographic capture mechanism can tolerate salt and urea in the urine sample that would otherwise need to be removed prior to mass spectrometric analysis. While neat urine can be analyzed directly on ProteinChip arrays, urine can also benefit from an enrichment step, which has been shown to increase the number of proteins detected more than twofold. Because urine volume and contents can vary substantially between individuals and within individuals over time, sample collection and storage should be carefully controlled to assure reproducible and clinically relevant results.

**Key words:** Urine, Protein profiling, SELDI, ProteinChip, ProteoMiner enrichment

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### 1. Introduction

Urine has been used for diagnosing disease for centuries, and urinalysis continues to be one of the most commonly used clinical tests. The non-invasive nature of sample collection makes urine an ideal source for diagnostic testing, particularly for indications that may require repeat sampling. Urine is also a relatively simple sample matrix compared to serum, containing less than 150 mg protein/day in healthy individuals. Normal urine volumes range from 0.6 to 2.0 L per day, with most people averaging between 1 and 1.5 L. Increases in total urinary protein, known as proteinuria, are indicative of disease and may result from a glomerular disease, low reabsorption rates in the proximal tubule, or an increase in proteins in serum. Proteinuria has been associated with a wide variety of diseases, including diabetes, pre-eclampsia, and multiple myeloma.

## Report Documentation Page

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The relatively low protein content of urine makes it an ideal sample for detecting perturbations in protein levels associated with disease. However, the low protein concentration and the high salt and urea concentrations encountered in urine make direct analysis challenging by most proteomics techniques. In contrast, SELDI is well suited for direct analysis of urine. Urine samples can be diluted in appropriate binding buffers and applied directly to chromatographic arrays. Salts and urea that interfere with mass spectrometric detection are subsequently washed off, yielding rich protein profiles. SELDI technology has been widely used to analyze urine for a wide variety of diseases, including diabetes (1, 2), renal allograft rejection (3–6), cancer (7–9), renal disease (10), and lupus (11).

While neat urine can be analyzed directly on ProteinChip arrays without pre-treatment, an up-front enrichment step can dramatically increase the number of proteins detected (12). The ProteoMiner technology is most widely used with serum, where it simultaneously reduces the concentration of high abundance proteins and enriches lower abundance proteins, ultimately increasing the number of proteins detected. This technology utilizes a combinatorial peptide ligand library. With sufficient peptide diversity, there should be a binding partner for most, if not all, proteins in a given sample. To achieve optimal function according to this design, the ProteoMiner resin is loaded far in excess of its protein-binding capacity. Under these competitive binding conditions, high affinity interactions with lower abundance proteins occupy more of the binding space on the resin, and most of the higher abundance proteins flow through. These ideal binding conditions are easily achieved when using voluminous samples with high protein concentrations like human serum. Achieving these conditions is more difficult with more dilute samples such as CSF and urine.

Despite the theoretical limitations of ProteoMiner technology when using small volumes of dilute samples, this method has proven extremely effective in increasing the number of protein species detected in clinically relevant volumes of urine. The method described here utilizes a sequential elution scheme, eluting bound proteins from the beads in three steps. Because the relatively high concentrations of salt and urea in urine prevent some proteins from binding to the peptide ligands, the flow through fraction should also be analyzed to maximize the number of proteins detected. Under these conditions, the observed increase in proteins after ProteoMiner treatment is likely a combination of enrichment and sample concentration, but has been shown to yield two to three-fold more protein peaks than analysis of neat urine alone.

While urine is a valuable medium for biomarker discovery, urine contents and volume can vary significantly between individuals and within individuals over time. It is therefore especially important to collect samples at the same time of day, using the same methods for collection and pre-storage processing (13).

Several different collection methods are possible, with the most frequently used collections being first and second morning void samples and 24-h urine. Other pre-analytical conditions should be carefully controlled, including the time urine is subjected to room temperature, the number of freeze thaw cycles, and denaturation steps (14). To reduce the impact of variability on statistical analysis, it is also advisable to analyze a larger number of patient samples than might be required for other biological fluids such as serum or CSF.

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## 2. Materials

### **2.1. Binding of Urine to ProteoMiner Resin**

1. 20% (v/v) Methanol in purified water (18 MΩ).
2. ProteoMiner resin, bulk dry (Bio-Rad).
3. Phosphate buffered saline.
4. 2-mL capacity, 96-well plates (Whatman), with silicone plate sealers.
5. Silent screen 96-well filter plate, loprodyn filter 1.2 μm pore (Nunc), and 96-well plates to collect washes.

### **2.2. Sequential Elution of Urine Proteins into Three Fractions**

1. TUC buffer: 7 M thiourea, 2 M urea, 4% CHAPS. Store aliquots of this buffer frozen (-20°C), after thawing discard the unused portion.
2. 96-well v-bottom collection plates (Nunc).
3. UCCit buffer: 9 M urea, 2% CHAPS, 25 mM citric acid – final pH should be ~3.55. Store aliquots of this buffer frozen (-20°C), after thawing discard the unused portion.
4. Organic solvent: 33% isopropyl alcohol, 16.7% acetonitrile, 0.1% trifluoroacetic acid. It is recommended that this volatile solvent be stored cold (4°C) and prepared fresh on a bi-weekly basis.

### **2.3. Profiling of Fractionated Urine by Retentate Chromatography and MALDI-TOF MS**

1. ProteinChip Arrays and bioprocessor apparatus (Bio-Rad).
2. Low stringency binding buffers corresponding to the arrays (Bio-Rad).
3. Sinapinic acid (Bio-Rad), dissolved in 400 μL 50% acetonitrile, 0.1% trifluoroacetic acid by shaking vigorously. Make fresh.

### **2.4. Profiling of Neat Urine by Retentate Chromatography and MALDI-TOF MS**

1. ProteinChip Arrays and bioprocessor apparatus (Bio-Rad).
2. Low stringency binding buffers corresponding to the arrays (Bio-Rad).
3. U9 buffer: 9 M urea, 2% CHAPS, 50 mM Tris-HCl pH 9.
4. Sinapinic acid (Bio-Rad), dissolved in 400 μL 50% acetonitrile, 0.1% trifluoroacetic acid by shaking vigorously. Make fresh.

### 3. Methods

Unlike other biological fluids like serum and CSF, urine can fluctuate significantly in terms of protein and salt concentration and can exhibit high variability both within a single patient over time and between individuals. Of the approximately 150 mg/day excreted in urine by healthy individuals, approximately 50% is Tamm-Horsfall mucoprotein (uromodulin) and another 10% is serum albumin. An increase in protein in the urine is itself indicative of pathological condition.

#### **3.1. Sample Collection and Storage**

1. Due to the high variability of urine over time and between individuals, it is especially important to collect samples at the same time of day and using the same methods.
  - (a) 24-h urine compensates for variations over the course of the day and generally yields the most consistent protein content. Where possible, 24-h urine is preferred due to its consistency.
  - (b) First and second morning voids are also frequently used. Both have relatively high protein concentrations. First morning voids generally have more particulate matter, and for this reason second morning voids are preferred when available.
2. Urine samples should be centrifuged prior to freezing at -80°C.

#### **3.2. Sample Preparation and Binding to ProteoMiner Resin**

1. Swell ProteoMiner beads overnight in 20% methanol. Wash swelled resin once in 5 volumes of water for 5 min with shaking. Equilibrate the beads in PBS by washing several times. Once equilibrated, suspend the beads in an equal volume of PBS to produce a 50% slurry.
2. Thaw urine samples on ice and centrifuge at 16,000 $\times g$  for 5 min at 4°C.
3. Add 40  $\mu$ L of the 50% ProteoMiner slurry (see Note 1) to each well of a multi-well plate (2 mL per well capacity).
4. Add 1.8 mL of urine sample to each well and seal using a silicone plate-sealer. Incubate the plate for 2 h at 4°C with gentle end-over-end rotation.
5. Centrifuge the sample plate 1,000 $\times g$  for 1 min to pellet the resin.
6. Aspirate about 1.6 mL of supernatant from each well and save as the Flow Through. Suspend the ProteoMiner beads in the remaining liquid and transfer to a filter plate.
7. Collect the rest of the Flow Through by placing the filter plate on top of a 96-well collection plate and centrifuge 1,000 $\times g$  for 1 min. Vacuum filtration may also be used if a centrifuge with a microplate adaptor is not available (see Note 2). Combine with the previously collected Flow Through.

- Suspend any remaining ProteoMiner beads in the sample plate in 200  $\mu$ L PBS and transfer to the filter plate. Shake for 5 min and collect the PBS wash by centrifuging at 1,000  $\times g$  for 1 min.
- Add 200  $\mu$ L PBS to each well of the filter plate, suspend the beads, and collect the wash by centrifugation. The wash may be discarded. Repeat this step three more times.

### 3.3. Sequential Elution of Urine Proteins into Fractions

Proteins can be eluted from ProteoMiner beads using a variety of different buffers. The method described below elutes proteins in three fractions, but other elution schemes can also be used and are described further in Note 3. Representative profiles of fractionated urine are shown in Fig. 1.

- Add 40  $\mu$ L of TUC buffer to each well of the filter plate and shake at room temperature for 15 min.
- Collect the eluate by centrifugation (1,000  $\times g$  for 1 min).
- Repeat steps 1 and 2, collecting the eluate in the same collection plate. This is Fraction 1.

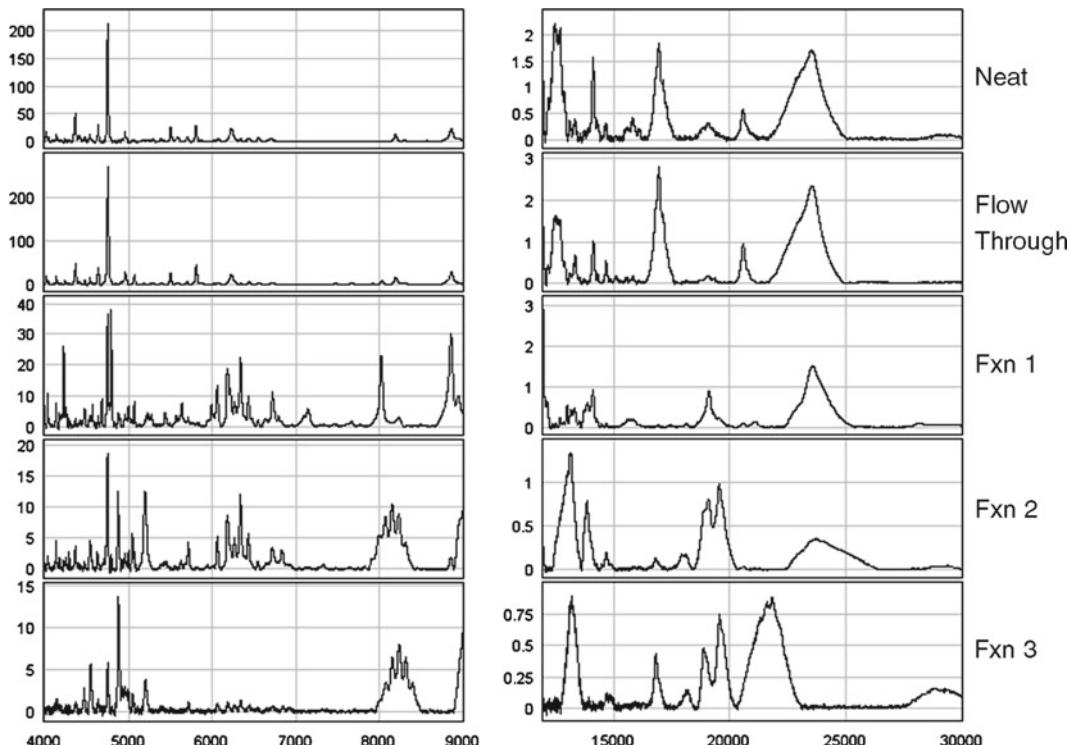


Fig. 1. ProteoMiner enrichment increases the number of peaks detected. 1.8 ml of urine was enriched and fractionated using ProteoMiner beads and the resulting fractions profiled on cation exchange (CM10) arrays. The number of peaks detected in the flow through and three eluates (201 peaks) was approximately 2.5-fold higher than the number detected in neat urine (81 peaks). Data is shown from the peptide range (4–9 kDa) and the protein range (12–25 kDa). Although urine contains relatively few high mass proteins, several proteins are detected only after enrichment.

4. Add 40  $\mu$ L of UCCit buffer to each well of the filter plate and shake at room temperature for 15 min.
5. Collect the eluate by centrifugation ( $1,000 \times g$  for 1 min) in a new collection plate. This is Fraction 2.
6. Repeat steps 4 and 5, collecting the eluate in the same collection plate.
7. Add 40  $\mu$ L of organic solvent to each well of the filter plate and shake at room temperature for 5 min.
8. Collect the eluate by centrifugation ( $1,000 \times g$  for 1 min) in a new collection plate.
9. Repeat steps 7 and 8, collecting the eluate in the same collection plate. This is Fraction 3.

### ***3.4. Profiling of Fractionated Urine by SELDI-TOF MS***

1. Assemble the appropriate number of ProteinChip arrays into a bioprocessor. Samples should be profiled in duplicate or triplicate. In addition to experimental samples, a reference sample should be profiled on every array to evaluate the reproducibility of the assay (Fig. 2).
2. Prepare the ProteinChip arrays for profiling. Fractionated urine should be profiled on multiple array chemistries to increase the total number of protein species detected. The most commonly used array chemistries are cation exchange (CM10), anion exchange (Q10), reverse phase (H50), and metal affinity (IMAC30). Representative spectra of neat urine profiles on these four array types are shown in Fig. 3.
  - (a) Equilibrate CM10 and Q10 arrays with the appropriate binding buffer by incubating twice for 5 min with 150  $\mu$ L of the appropriate binding buffer on a plate shaker.
  - (b) Pre-treat H50 arrays with 50  $\mu$ L of 50% acetonitrile for 5 min and allow arrays to dry. Equilibrate by incubating twice for 5 min with 150  $\mu$ L of H50 binding buffer on a shaker.
  - (c) Charge IMAC30 arrays with copper by adding 50  $\mu$ L of IMAC charging solution and incubating 10 min on a shaker. Remove charging solution and wash once with 150  $\mu$ L water, incubating 1 min on a shaker. Add 150  $\mu$ L neutralization buffer, incubating 5 min on a shaker. Remove neutralization solution and wash once with 150  $\mu$ L water, incubating 1 min on a shaker. Remove water and equilibrate by incubating with IMAC buffer twice for 5 min each on a shaker.
3. Once the chromatographic surface has been equilibrated, add 90  $\mu$ L binding buffer to each well of the bioprocessor. Add 10  $\mu$ L of the fraction to be profiled (see Note 4). Shake 30 min at room temperature.

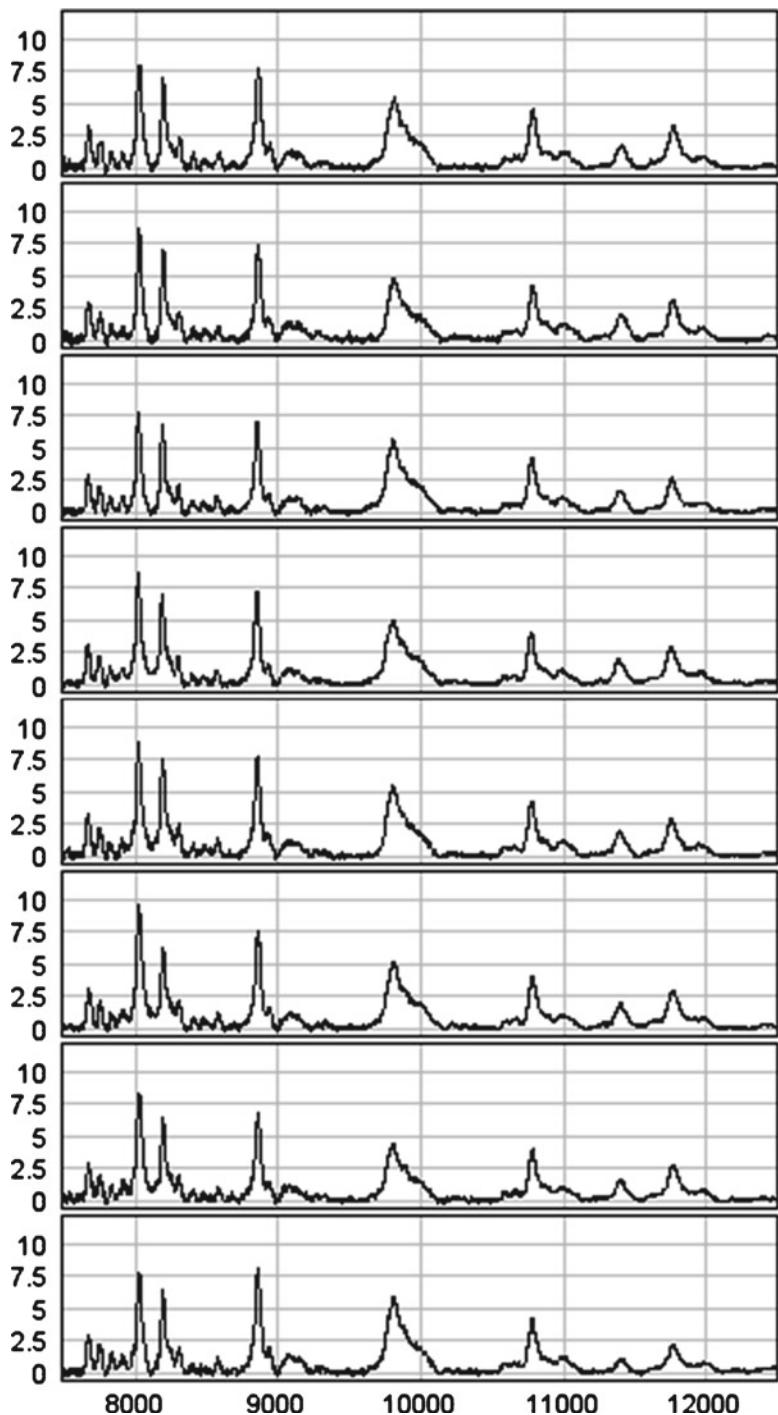


Fig. 2. Reproducibility of urine profiling. A total of 24 aliquots of urine were analyzed alongside experimental samples (one spot on each of 24 ProteinChip arrays) and used to assess assay reproducibility. The data shown here was collected on cation exchange arrays and yielded a median CV of 16.7%. The mass range from 7.5 to 12.5 kDa is shown.

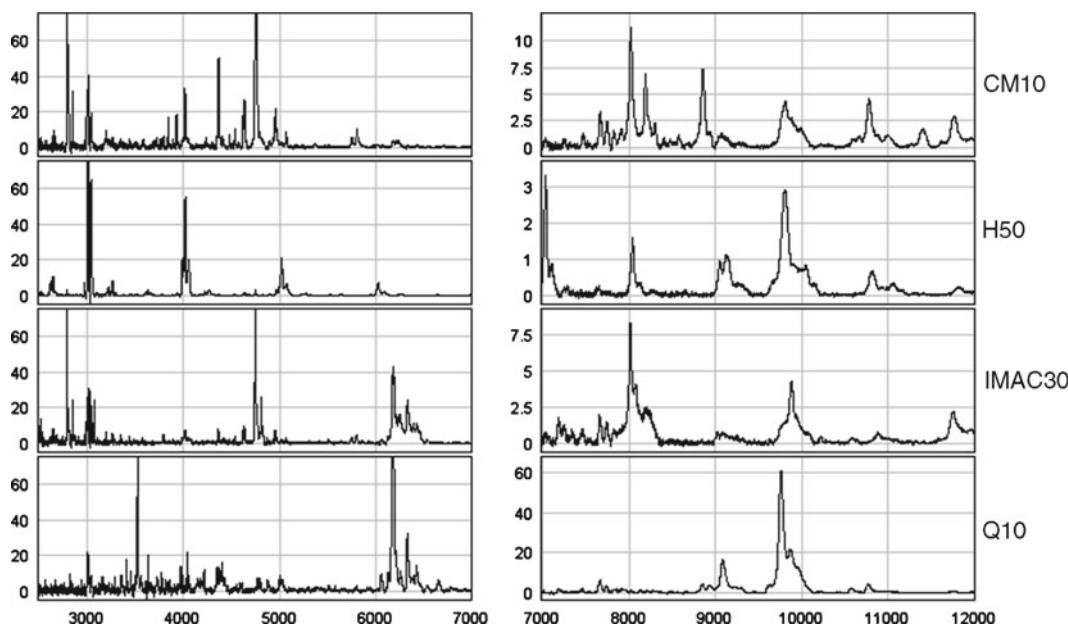


Fig. 3. Profiling on multiple array surfaces increases the number of peaks detected. Neat urine was profiled on four different ProteinChip array chemistries, including cation exchange (CM10), reverse phase (H50), copper affinity (IMAC30-Cu), and anion exchange (Q10). While some proteins bind to multiple surfaces, each surface captures a unique subset of proteins, thereby increasing the total number of peaks detected. Data is shown from two different mass ranges (2.5–7 kDa and 7–12 kDa).

4. Aspirate and discard the liquid from each well. Wash each spot with 150  $\mu$ L binding buffer for 5 min with vigorous shaking. Repeat this step two more times.
5. Briefly rinse metal affinity and ion exchange arrays with 200  $\mu$ L of HPLC grade water. Hydrophobic arrays do not require a water wash. Repeat this step. Allow the arrays to dry for 10 min.
6. Apply 1  $\mu$ L of 12.5 mg/mL SPA (see Note 5) to each spot and allow to dry for 10 min. Repeat this step once.
7. Analyze by TOF MS.

### 3.5. Profiling of Neat Urine and the ProteoMiner Flow Through Fraction

In the event that sufficient sample volumes are not available to utilize ProteoMiner fractionation, urine samples may be profiled without fractionation. Because urine samples (particularly 24-h samples) are often quite dilute, multiple loadings may be beneficial. As with fractionated urine, samples should be profiled on multiple array types, usually including reverse phase (H50), metal affinity (IMAC30), anion (Q10) and cation (CM10) exchange. Neat urine samples should also be denatured prior to profiling to improve reproducibility and minimize variability between samples.

### 3.5.1. Sample Preparation

1. Centrifuge urine at  $16,000 \times g$  for 5 min at 4°C.
2. Denature urine by mixing 110  $\mu$ L of urine with 175  $\mu$ L of U9 buffer.
3. Incubate for 30 min at 4°C.

### 3.5.2. Profiling

1. Assemble the appropriate number of ProteinChip arrays into a bioprocessor. Samples should be profiled in duplicate or triplicate. In addition to experimental samples, a reference sample should be profiled on every array.
2. Prepare the ProteinChip arrays for profiling. Neat urine should be profiled on multiple array chemistries to increase the total number of protein species detected. The most commonly used array chemistries are reverse phase (H50), metal affinity (IMAC30), and anion (Q10) and cation (CM10) exchange.
  - (a) Equilibrate CM10 and Q10 arrays with binding buffer by incubating twice for 5 min with 150  $\mu$ L of the appropriate binding buffer on a plate shaker.
  - (b) Pre-treat H50 arrays with 50  $\mu$ L of 50% acetonitrile for 5 min and allow arrays to dry. Equilibrate by incubating twice for 5 min with 150  $\mu$ L of H50 binding buffer on a shaker.
  - (c) Charge IMAC30 arrays with copper by adding 50  $\mu$ L of IMAC charging solution and incubating 10 min on a shaker. Remove charging solution and wash once with 150  $\mu$ L water, incubating 1 min on a shaker. Add 150  $\mu$ L neutralization buffer, incubating 5 min on a shaker. Remove neutralization solution and wash once with 150  $\mu$ L water, incubating 1 min on a shaker. Remove water and equilibrate by incubating with IMAC buffer twice for 5 min each on a shaker.
3. Once the chromatographic surface has been equilibrated, add 175  $\mu$ L binding buffer to each well of the bioprocessor. Add 25  $\mu$ L of the neat urine. Shake vigorously for 30 min at room temperature (see Note 6).
4. Aspirate and discard the liquid from each well. Wash each spot with 150  $\mu$ L binding buffer for 5 min with vigorous shaking. Repeat this step two more times.
5. Briefly rinse metal affinity and ion exchange arrays with 200  $\mu$ L of HPLC grade water. Hydrophobic arrays do not require a water wash. Repeat this step.
6. Allow the arrays to dry for 10 min.
7. Apply 1  $\mu$ L of 12.5 mg/mL SPA to each spot and allow to dry for 10 min. Repeat this step once.
8. Analyze by TOF MS.

## 4. Notes

1. When dispensing aliquots of 50% ProteoMiner bead slurry, vortex the slurry frequently to maintain an even suspension during pipetting.
2. If a centrifuge with a microplate adaptor is not available, a vacuum manifold may also be used for fractionation. Typically, vacuum is applied for 1–2 min at each step. The filtration should be sufficient to remove all liquid from the resin, but overdrying should be avoided since it can make subsequent elution more difficult.
3. A number of different elution schemes can be utilized and selection of the appropriate method depends, in part, on the downstream analysis methods. To elute proteins in a single step, use either UCCit (9 M urea, 2% CHAPS, 25 mM citric acid), or 8 M urea in 5% acetic acid. Two alternative sequential elution schemes are described below. Fractionation using these alternative methods can be performed as described in Subheading 3.

(a) Scheme 1

Fraction 1: 1 M NaCl, 20 mM HEPES pH 7.5
Fraction 2: 0.2 M Glycine pH 2.4
Fraction 3: 60% ethylene glycol
Fraction 4: Organic solvent (33% isopropyl alcohol, 16.7% acetonitrile, 0.1% trifluoroacetic acid)

(b) Scheme 2

Fraction 1: 1 M NaCl, 20 mM HEPES pH 7.5
Fraction 2: 3 M Guanidine–HCl, 25 mM MES pH 6
Fraction 3: UCCit buffer (9 M urea, 2% CHAPS, 25 mM citric acid)

4. To improve uniformity of data, it is important to avoid bubbles over the chromatographic spots at the bottom of the wells. Bubbles can be effectively removed by centrifuging the bio-processor  $250 \times g$  for 1 min after addition of buffer.
5. Sinapinic acid is the most frequently used matrix for SELDI-based protein profiling since it allows visualization of the widest mass range. However, other matrices commonly used for protein mass spectrometry may also be used. The second most commonly used matrix for this purpose is  $\alpha$ -Cyano-4-hydroxycinnamic acid (CHCA), which is typically made as a 25–50% saturated solution in 0.1% TFA, 50% acetonitrile.

The percentage saturation may need to be adjusted for different mass spectrometers and should be optimized on a reference sample prior to analyzing experimental samples.

6. In some cases, data quality can be improved by applying a second 25  $\mu$ L of urine to increase protein load. This is especially important for more dilute samples such as 24 h urine.

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